Role of TPBG (Trophoblast Glycoprotein) Antigen in Human Pericyte Migratory and Angiogenic Activity

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Objective—To determine the role of the oncofetal protein TPBG (trophoblast glycoprotein) in normal vascular function and reparative vascularization.

Approach and Results—Immunohistochemistry of human veins was used to show TPBG expression in vascular smooth muscle cells and adventitial pericyte-like cells (APCs). ELISA, Western blot, immunocytochemistry, and proximity ligation assays evidenced a hypoxia-dependent upregulation of TPBG in APCs not found in vascular smooth muscle cells or endothelial cells. This involves the transcriptional modulator CITED2 (Atypical chemokine receptor 3 CBP/p300-interacting transactivator with glutamic acid (E)/aspartic acid (D)-rich tail) and downstream activation of CXCL12 (chemokine [C-X-C motif] ligand-12) signaling through the CXCR7 (C-X-C chemokine receptor type 7) receptor and ERK1/2 (extracellular signal-regulated kinases 1/2). TPBG silencing by siRNA transfection downregulated CXCL12, CXCR7, and pERK (phospho Thr202/Tyr204 ERK1/2) and reduced the APC migratory and proangiogenic capacities. TPBG forced expression induced opposite effects, which were associated with the formation of CXCR7/CXCR4 (C-X-C chemokine receptor type 4) heterodimers and could be contrasted by CXCL12 and CXCR7 neutralization. In vivo Matrigel plug assays using APCs with or without TPBG silencing evidenced TPBG is essential for angiogenesis. Finally, in immunosuppressed mice with limb ischemia, intramuscular injection of TPBG-overexpressing APCs surpassed naïve APCs in enhancing perfusion recovery and reducing the rate of toe necrosis.

Conclusions—TPBG orchestrates the migratory and angiogenic activities of pericytes through the activation of the CXCL12/CXCR7/pERK axis. This novel mechanism could be a relevant target for therapeutic improvement of reparative angiogenesis.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2019;39:1113-1124. DOI: 10.1161/ATVBAHA.119.312665.)**

Key Words: aspartic acid ■ endothelial cells ■ glycoproteins ■ immunohistochemistry ■ pericytes

The TPBG (trophoblast glycoprotein, HGNC:12004) is a heavily N-glycosylated transmembrane protein belonging to the leucine-rich repeat family of proteins. It is typically expressed during fetal development and cancer.[1] In murine embryonic stem cells and fibroblasts, and cancerous cells, TPBG upregulation has been associated with the induction of the CXCL12 (chemokine [C-X-C motif] ligand-12). In addition, TPBG prolongs CXCL12 bioavailability via the inhibition of DPP4 (dipeptidyl peptidase-4), a protease responsible for CXCL12 cleavage.[1–3] Mechanistically, the TPBG/CXCL12 duo exert a modulatory control of gene transcription, proliferation, and directional movement of cancerous cells.[1,4–6] CXCL12 triggers intracellular signaling by binding to CXCR4 (C-X-C chemokine receptor-4) as well as to the noncanonical CXCR7 (C-X-C chemokine receptor-7) and CXCR4/CXCR7 heterodimers. The activated CXCR4 induces intracellular G-coupled protein cell signaling and is degraded after CXCL12-elicited internalization.[6] In contrast, CXCR7 becomes dominant after TPBG downregulation, signals through G-coupled protein independent mechanisms, and functions, at least, in part, as a chemokine decoy receptor for CXCL12.[3,7–11] Interestingly, both CXCR4 and CXCR7 can also form complexes with β-arrestin-2, thereby promoting receptor internalization and redirecting signaling to alternative G protein-independent pathways. For instance, β-arrestin-2 is reportedly involved in CXCR4- and CXCR7-mediated chemotaxis, and this is mediated by p38 MAPK/ERK (p38 mitogen-activated protein kinase/extracellular signal–regulated kinases) activation.[12,13]

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Nonstandard Abbreviations and Acronyms

APCs	adventitial pericytes
CCM	cell conditioned media
CITED2	Atypical chemokine receptor 3 CBP/p300-interacting transactivator with glutamic acid (E)/aspartic acid (D)-rich tail
CXCL12	chemokine (C-X-C motif) ligand-12
CXCR4	C-X-C chemokine receptor type 4
CXCR7	C-X-C chemokine receptor type 7
DPP4	dipeptidyl-peptidase-4
EGM2	endothelial growth medium 2
EMT	epithelial-to-mesenchymal transition
HIF	hypoxia-inducible factor
HUVECs	human umbilical cord endothelial cells
MMPs	matrix metalloproteinases
PDGFR-β	platelet-derived growth factor receptor-β
PLA	proximity ligation assay
siTPBG	TPBG siRNAs
SNAI	snail family transcriptional repressor
TPBG	trophoblast glycoprotein
TWIST1	twist family BHLH transcription factor 1
VSMCs	vascular smooth muscle cells
ZEB	zinc finger E-box binding homeobox

TPBG is also considered an early marker of the epithelial-to-mesenchymal transition (EMT) and the cognate endothelial-to-mesenchymal process, along with MMPs (matrix metalloproteinases), the E-cadherin repressors SNAI (Snail Family Transcriptional Repressor) 1 and 2, and the transcriptional factors ZEB (zinc finger E-box binding homeobox) and TWIST (twist family BHLH transcription factor) 1.[14–17] Cancerous cells undergoing EMT manifest multiple biochemical changes that enable them to acquire enhanced migratory capacity, invasiveness, and elevated resistance to apoptosis.[18] Furthermore, recent studies showed a similar transition confers cancerous cells with the characteristics of pericytes, thereby favoring vascular remodeling, sustained tumor growth, and increased invasiveness.[19,20] However, to the best of our knowledge, no data are currently available regarding a possible role of TPBG in postnatal vascular repair.

The present study investigates the potential role of TPBG in pericyte biology. In particular, we focus on a subset of pericyte-like cells isolated from the adventitial vasa vasorum, otherwise known as adventitial pericytes (APCs).[21–23]

Materials and Methods

All data and supporting materials have been provided with the published article (The authors declare that all supporting data are available within the article [and its online supplementary files]). The article adheres to the American Heart Association Journals Guidelines Implementation of the Transparency and Openness Promotion. All studies performed on human tissues and cells followed the ethical guidelines of the Declaration of Helsinki. Animal experiments were performed in accordance with the Animal (Scientific Procedures) Act (United Kingdom) 1986 prepared by the Institute of Laboratory Animal Resources and under the auspices of UK Home Office Project and Personal License. Results are reported following the guidelines contained in the Animal Research Report of In Vivo Experiments.

Commercial human umbilical cord endothelial cells (HUVECs, Lonza) or aortic vascular smooth muscle cells (VSMCs, PromoCell) have been cultured according to provider's instructions, while human APCs and murine CD31 or CSPG4 were immunomagnetic isolated from human veins or ischemic limbs, respectively, according to previous publications.[21,24]

Forced *TPBG* overexpression experiments were performed using adenoviral constructs. Adenoviral titration was calculated according to previous publications.[25] PcDNA3-*CITED2*-hemagglutinin was from Shoumo Bhattacharya[26] used to study effect of CITED2 (Atypical chemokine receptor 3 CBP/p300-interacting transactivator with glutamic acid (E)/aspartic acid (D)-rich tail) overexpression on TPBG. Additional ChIP assays performed as previously described[27] were used to evidence CITED2-*TPBG* binding.

Refer to Material in the online-only Data Supplement for further information.

Results

Expression of TPBG in Human Veins and Vascular Cells

To identify the localization of TPBG, we performed multicolor immunohistochemistry staining of human veins. Results indicate TPBG is expressed by VSMCs in the tunica media and CD34/CSPG4 positive APCs in proximity of the adventitial vasa vasorum (Figure IA and IB in the online-only Data Supplement). Flow cytometry of culture-expanded APCs confirmed TPBG was expressed by >95% of the cells, together with pericyte and mesenchymal markers, such as CD105, CD90, CD44, CSPG4, and PDGFR (platelet-derived growth factor receptor)-β. As expected, APCs were negative for CD31, CD146, and CD45 (Figure IC and ID in the online-only Data Supplement). We next compared the TPBG transcript levels in APCs, HUVECs, and proliferative or contractile VSMCs (Figure IE in the online-only Data Supplement). In sequence, proliferative VSMCs showed the highest number of *TPBG* transcript copies (10 135±2066, $P<0.01$ versus APCs), followed by contractile VSMC (7003±966, $P=0.85$ versus APCs), APCs (6889±661, $P<0.001$ versus HUVECs), and HUVECs (563±292). Positive and negative controls for the antibody and fluorescent technique are shown in Figure IIA and IIB in the online-only Data Supplement.

TPBG expression has been associated with migratory/invasive profiles in cancer cells.[18] Here, we show that TPBG expression is influenced by changes in APC confluence, with sparse cells showing higher TPBG levels than confluent cells (Figure IIIA through IIIC in the online-only Data Supplement). In addition, an in vitro scratch assay showed that physical discontinuation of APC monolayers increased the expression of TPBG, with positive staining being evident in the filopodia at the wound edges (Figure IIID and IIIE in the online-only Data Supplement). To the best of our knowledge, this is the first demonstration of TPBG being expressed by mural cells in adult human blood vessels and associated with cell motility.

Hypoxia Induces TPBG Expression in Human APCs

We next asked whether hypoxia may induce TPBG in APCs. In line with this possibility, hypoxia increased *TPBG* transcript levels compared with normoxia (1.6-fold at 12 hours and 2.0-fold at 24 hours, $P<0.05$ for both comparisons). *TPBG*

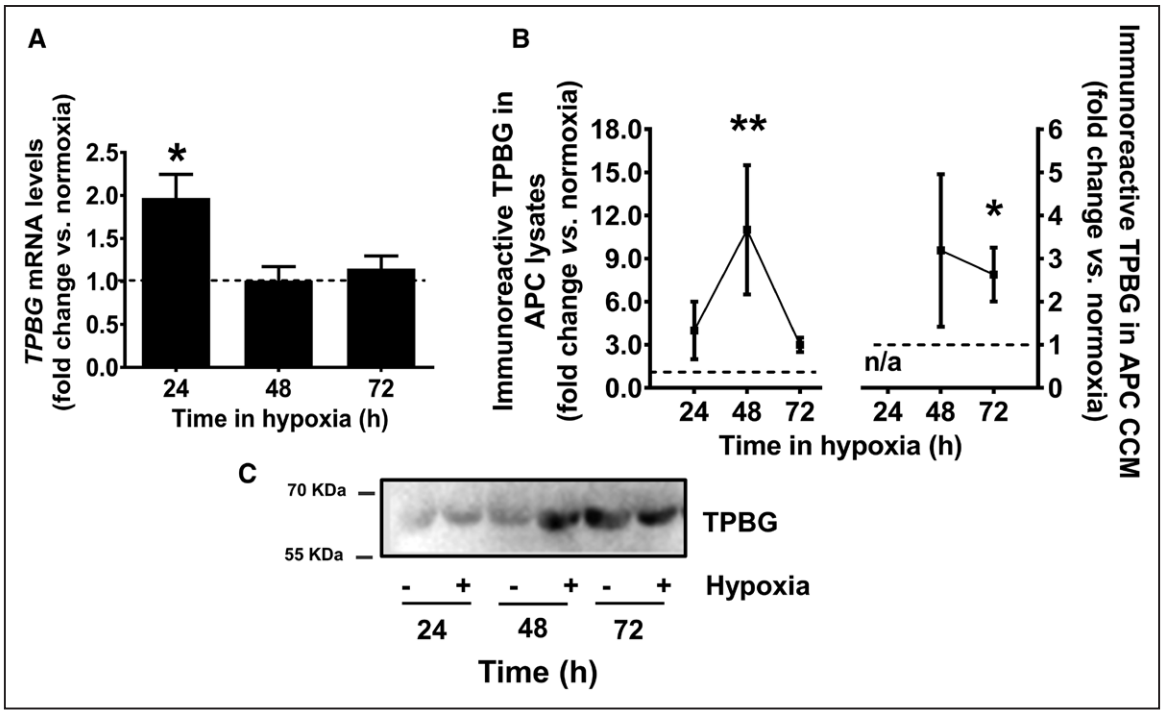

Figure 1. Hypoxia induces *TPBG* expression in human adventitial pericytes (APCs). **A**, Hypoxic-dependent modulation of TPBG (trophoblast glycoprotein) in 3 APC lines. Data are fold changes (mean±SEM) at the corresponding normoxia time points. Unpaired *t* tests were used to compare hypoxia vs normoxia; **P*<0.05. **B**, TPBG levels were assessed by ELISA in lysates and conditioned media (CCM) of the same APC lines. Data are fold changes (mean±SEM) vs corresponding normoxia time points. Not assessed (na). Unpaired *t* tests were used to compare hypoxia vs normoxia; **P*<0.05 and ***P*<0.01. **C**, Representative time course of TPBG protein expression (Western blot) in APC CCM to validate ELISA findings.

mRNA returned to baseline under prolonged hypoxia conditioning (Figure 1A). In contrast, neither HUVECs nor VSMCs (3 cell lines each) exhibited *TPBG* regulation after hypoxic conditioning (data not shown). We verified that immunoreactive TPBG protein is modulated by hypoxia in APC lysates (*P*<0.01 versus normoxia) and cell conditioned media (CCM; *P*<0.05 versus normoxia) using ELISA assays (Figure 1B). The TPBG content in the secretome was further assessed by Western blotting, using a different anti-TPBG antibody to verify the ELISA results (Figure 1C). Proximity ligation assay (PLA) was used for target validation and antibody specificity (Figure IV in the online-only Data Supplement), also confirming the modulation of TPBG by hypoxia (Figure IVA and IVD in the online-only Data Supplement).

As stated above, TPBG is considered an EMT marker. Accordingly, the modulation of TPBG by hypoxia was associated with a concordant induction of the EMT transcription factors *TWIST*, *SNAI1*, *SNAI2*, and *ZEB2* (*P*<0.05 versus normoxia for all comparisons; Figure VA through VD in the online-only Data Supplement). In contrast, the levels of *TIMP1* and *TIMP2* were temporarily decreased (*P*<0.05 and *P*<0.01 after 24 hours of hypoxia; Figure VE and VF in the online-only Data Supplement). MMPs followed discordant patterns, with *MMP2* transcripts and gelatinolytic activity being increased (*P*<0.05 versus normoxia; Figure VG and VH in the online-only Data Supplement), and *MMP9* transcripts remaining unchanged (data not shown). To verify the dependency of the above EMT markers on TPBG, we measured their expression following *TPBG* silencing by siRNA. Results indicate *SNAI2*, *TIMP1,* and *TIMP2* were all suppressed by the TPBG inhibition in normoxic or hypoxic APCs (Figure VI and VN in the online-only Data Supplement).

Hypoxia Induces the CXCL12/CXCR7 Axis in Human APCs

Previous investigation has shown that TPBG is required for CXCL12-mediated chemotaxis via ligand interaction with either CXCR4 or CXCR7 receptors in embryo fibroblasts and cancerous cells.[1,3] Interestingly, we found that hypoxic-dependent induction of TPBG was associated with an increased expression and secretion of CXCL12 by human APCs (*P*<0.05 versus normoxia; Figure 2A and 2B). No significant changes were found about other cytokines implicated in chemotaxis (such as CCL2/MCP1, CXCL2/GRO β [C-X-C motif chemokine 2/growth-regulated protein beta], and CXCL1/GRO-α [C-X-C motif chemokine 1/growth-regulated alpha protein]; data not shown).[28,29]

In addition, immunocytochemistry (ICC) and PLA techniques showed a differential pattern of expression and subcellular localization for CXCR4 and CXCR7 receptors (Figure 2C and 2D). In normoxic APCs, CXCR4 prevailed over CXCR7, whereas following hypoxia CXCR4 acquired a prevalent nuclear localization and CXCR7 became upregulated. The latter result was confirmed by measurement of CXCR7 mRNA levels, which showed a 4.2-fold increase versus normoxia (*P*<0.01; data not shown). Similar changes in the repertoire and subcellular localization of CXCL12 receptors has been associated with the migratory phenotype of metastatic cells.[1,30] But never reported previously in vascular pericytes. The PLA experiment illustrated in Figure 2D shows additional features of molecular changes induced by

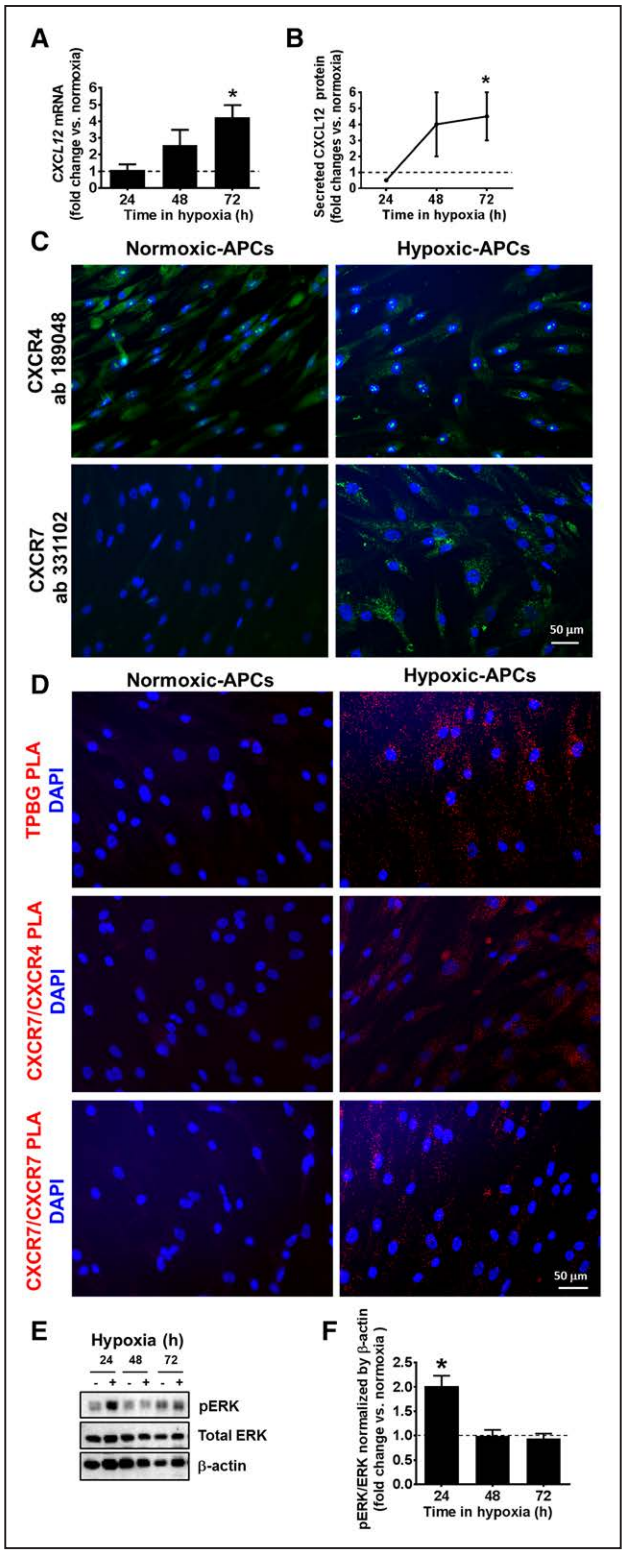

Figure 2. Hypoxic-dependent regulation of the CXCL12 (chemokine [C-X-C motif] ligand-12) signaling in human adventitial pericytes (APCs). **A**–**D**, The modulation of the CXCL12/CXCR4 (C-X-C chemokine receptor type 4)/CXCR7 (C-X-C chemokine receptor type 7) axis by hypoxia was studied in 4 to 5 APC lines. Hypoxia increases the levels of CXCL12 at mRNA (**A**) and immunoreactive protein level (**B**). Data are fold changes (mean±SEM) vs corresponding normoxia time points. Unpaired *t* tests were used to compare hypoxia vs normoxia in 5 APC lines, **P*<0.05. Representative images of immunohistochemistry (**C**) and proximity ligation assay (PLA; **D**) from studies performed in 4 APC (*Continued*)

hypoxia: first, it confirmed at the molecular level that hypoxia induced the candidate target TPBG (upper); second, it verified the concomitant upregulation of CXCR7 (lower); and third, using antibodies against CXCR4 and CXCR7, it demonstrated that hypoxia caused the formation of receptor heterodimers (middle). In contrast, no PLA signals were detected when only one primary antibody was used or when both primary antibodies were omitted (data not shown).

We also measured the expression of DPP4, one pivotal CXCL12 degrading protease, and found it to be downregulated at the transcript and protein level after 72 hours of hypoxia (0.68±0.11 *DPP4* mRNA fold change versus normoxia, *P*<0.05; 0.53±0.09 DPP4 secreted fold change, *P*<0.001 versus normoxia). Altogether, these data indicate hypoxia has a profound impact on different components of the CXCL12 pathway, which would be expected to result in the activation of downstream kinase signaling. In line with this, we found that hypoxic conditioning of APCs induces the phosphorylation of ERK (Figure 2E and 2F), which is a typical target of CXCL12 binding to CXCR7.[6]

Forced Modulation of TPBG Expression Alters APC Functional Behavior Through the CXCL12/CXCR7/pERK Signaling Pathway

The functional implications of the TPBG/CXCL12 partnership were further investigated by silencing or overexpressing *TPBG* in human APCs under normoxia or hypoxia.

In silencing experiments, normoxic or hypoxic APCs were transfected with *TPBG* siRNAs (siTPBG) or scramble controls (siCtrl). The latter did not alter the inductive effect of hypoxia on TPBG, CXCL12, and CXCR7 expression (Figure 3A through 3D). siTPBG efficiently reduced the transcript and protein levels of TPBG in normoxic or hypoxic APCs, as demonstrated by quantitative polymerase chain reaction (qPCR), Western blot, and PLA (Figure 3A and 2B, *P*<0.01 versus siCtrl; Figure IVB and IVD in the online-only Data Supplement).

Next, we investigated whether TPBG silencing would affect the APC behavior. Previous studies have attributed TPBG with the properties of an early differentiation marker in mouse embryonic stem cells, and its absence may be a useful mean to assess pluripotency.[14] APCs coexpress typical pericyte/stromal cell markers together with stemness antigens.[21] Therefore, we verified the effect of siTPBG on the APC antigenic profile and found no change as compared with siCtrl-treated APCs (data not shown). Similarly, siTPBG did not cause any effect on proliferation or apoptosis (data not shown). Looking at CXCL12 expression and CXCL12-associated functions, we found that, in normoxic APCs, siTPBG reduced CXCL12 (Figure 3E and 3F), CXCR7 (Figure 3G and 3H), and pERK (Thr202/Tyr204; Figure 3I and 3J), with these effects being associated with diminished migratory activity in a scratch assay (0.66±0.03 gap closure fold change, *P*<0.01 versus siCtrl) and impaired capacity to support endothelial

Figure 2 Continued. lines to verify the expression and subcellular localization of TPBG (trophoblast glycoprotein), CXCR4, and CXCR7. **E** and **F**, Hypoxia induces the phosphorylation of ERK (Thr202/Tyr204; extracellular signal-regulated kinases 1/2). Representative Western blot images (**E**) and results of densitometry (**F**). Data are fold changes (mean±SEM) vs corresponding normoxia time points in 4 APC lines. Unpaired *t* test, **P*<0.05.

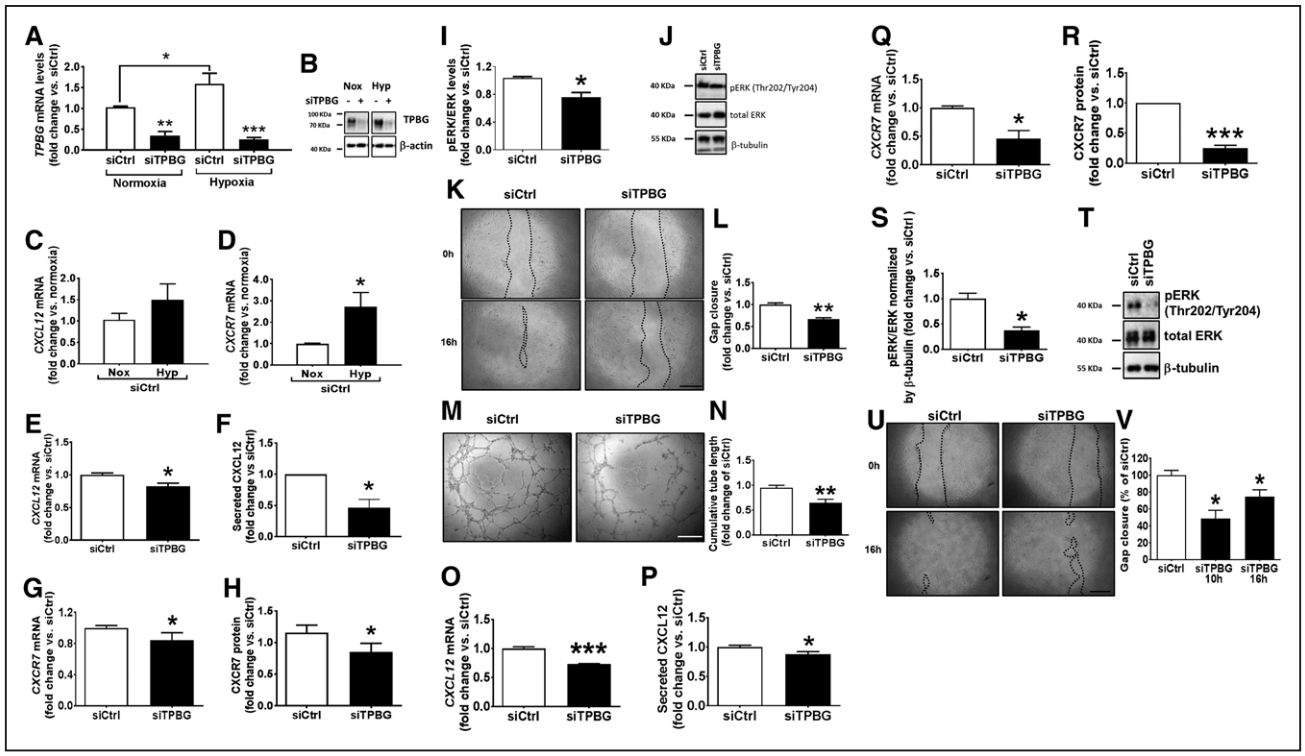

Figure 3. *TPBG* silencing inhibits CXCL12 (chemokine [C-X-C motif] ligand-12) signaling and impairs migration and network forming activity. **A** and **B**, The effective silencing of *TPBG* by siRNA was demonstrated by quantitative polymerase chain reaction (qPCR; **A**) and Western blot (**B**) in 3 adventitial pericytes (APC) lines under normoxic or hypoxic conditions. **C** and **D**, Bar graphs showing that hypoxia-induced upregulation of CXCL12 and CXCR7 (C-X-C chemokine receptor type 7) was not altered in control APCs treated with siRNA control (siCtrl). **E**–**J**, *TPBG* silencing in normoxic APCs (4 cell lines) reduced the levels of *CXCL12* mRNA (**E**) and secreted CXCL12 protein (**F**) as well as *CXCR7* mRNA (**G**), CXCR7 protein (**H**), and pERK (**I** and **J**). **K** and **L**, In a scratch assay on 3 APC lines, TPBG (trophoblast glycoprotein) silencing impaired gap closure. **M** and **N**, In a Matrigel assay using 5 APC lines, siRNA TPBG (siTPBG) reduced the cell's ability to support an endothelial network. Scale bars, 500 μm. **O**–**V**, TPBG silencing in hypoxic APCs (4 to 5 cell lines) produced similar effects, reducing *CXCL12* mRNA (**O**) and secreted CXCL12 protein (**P**), as well as *CXCR7* mRNA (**Q**), CXCR7 protein (**R**), and pERK (**S** and **T**). In addition, siTPBG reduced gap closure (**U** and **V**) in 3 APC lines. Scale bars, 500 μm. Data are the mean±SEM. ANOVA was used to compare multiple groups and unpaired *t* tests to compare 2 groups, **P*<0.05, ***P*<0.01, and ****P*<0.001 vs siCtrl. White bars, siCtrl in normoxia (Nox) conditions; green bars, siTPBG in Nox conditions; black bars, siCtrl in hypoxic (Hyp) conditions; red bars, siTPBG in Hyp conditions.

network formation on Matrigel (0.65±0.07 tube length fold change, *P*<0.01 versus siCtrl; Figure 3K through 3N). In hypoxic APCs, siTPBG was once again associated with lowered levels of the CXCL12, CXCR7, and pERK (Figure 3O through 3T) and diminished migratory activity (Figure 3U and 3V). No effect of silencing was observed on other chemokines, such as CCL2/MCP1, CXCL2/GRO, and CXCL1, or DPP4 in normoxic or hypoxic conditions (data not shown).

We next assessed the impact of transfecting APCs with Ad-*TPBG*; the empty backbone Ad-Null was used as a control (Figure 4). These experiments were performed under normoxic conditions to verify if forced *TPBG* expression could phenocopy the effects observed under hypoxia. Effective transduction was evidenced by qPCR, Western blot, PLA, and ICC (Figure 4A and 4B; Figures IVC and IVD and VI in the online-only Data Supplement). In addition, we found that *TPBG*-transduced APCs retained the capacity to upregulate the endogenous protein following exposure to hypoxia (Figure VID in the online-only Data Supplement).

Forced *TPBG* expression was associated with increased CXCL12 secretion into the culture media (1.38±0.01-fold, *P*<0.001 versus Ad-Null; Figure 4C). Looking at the CXCL12 receptors, we could not observe any effect of Ad-*TPBG* on the expression levels and subcellular localization of CXCR4 and CXCR7, as assessed by ICC (Figure 4D, upper). Therefore, the nuclear import of CXCR4 and upregulation of CXCR7 seem to be a unique prerogative of APCs exposed to hypoxia. On the contrary, PLA demonstrated that *TPBG*-transduced APCs carry CXCR7/CXCR4 heterodimers as also seen in hypoxic APCs (Figure 4D, lower). In the scratch assay, *TPBG*-transduced APCs showed a 5.4-fold increase in gap closure compared with Ad-Null treated APCs (*P*<0.01, Figure 4E and 4F). Finally, we examined the effect of forced *TPBG* expression on the APCs' interaction with HUVECs in a Matrigel assay. The CCM from TPBG-transduced APCs did not promote any increase in network formation as compared with the CCM from Null-transfected APCs (0.94±0.02-fold change; data not shown). Likewise, TPBG-transduced APCs in coculture with HUVECs caused a modest increase in the length of the networks, which did not reach the statistical significance (1.16±0.11-fold change versus Ad-Null, *P*=0.28; data not shown). In contrast, TPBG-transduced APCs could increase the HUVEC network formation when the 48 hours CCM from the same TPBG-transduced APCs was added to the coculture (1.34±0.07-fold change, *P*<0.01 versus Ad-Null; Figure 4G and 4H). This latter data suggest cumulative effects of APCs and their CCM are responsible for the increased in vitro angiogenic improvement.

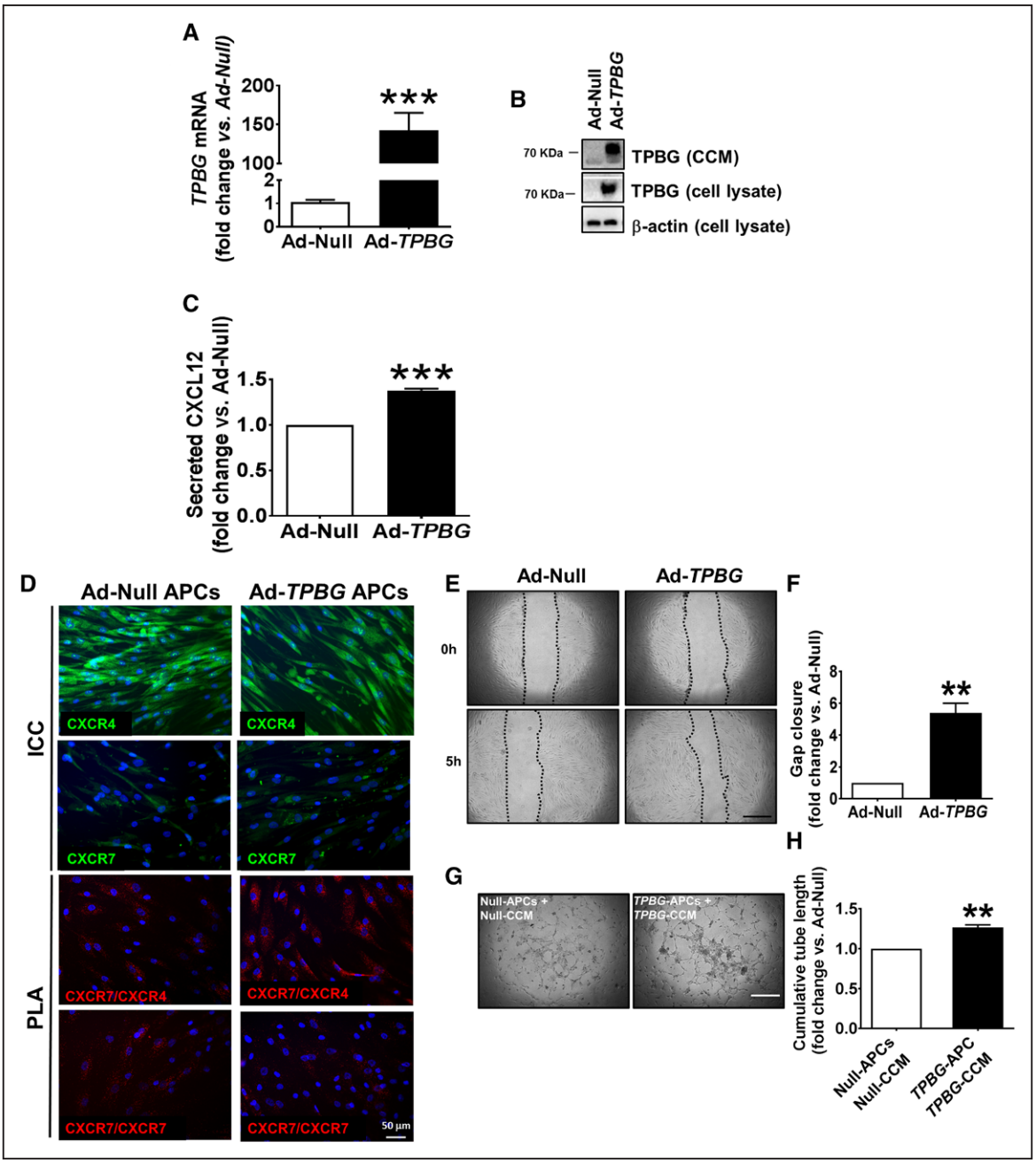

Figure 4. Forced *TPBG* expression activates the CXCL12 (chemokine [C-X-C motif] ligand-12) signaling and promotes migration and network forming activity. Adventitial pericytes (APCs) were treated with either Null vector or a *TPBG* expression Ad vector under normoxic conditions. Effective transduction was confirmed in 3 APC lines by quantitative polymerase chain reaction (qPCR; **A**, Western blotting in cell conditioned medium (CCM) and cell lysate (**B**) after 24 h from the transfection. Forced TPBG (trophoblast glycoprotein) expression was associated with increased secretion of CXCL12 (**C**) and complexing of the CXCR4 (C-X-C chemokine receptor type 4) receptors as seen by proximity ligation assay (PLA; **D**). **E** and **F**, Forced expression of *TPBG* enhances the migration of APCs (4 cell lines) in a scratch assay. Representative microscopy images (**E**) and bar graphs showing fold changes vs Null (**F**). **G** and **H**, Matrigel assay in APCs (5 cell lines) treated with either Null or *TPBG* Ad vector and cocultured with human umbilical cord endothelial cells (HUVECs) for assessment of network formation in the presence of Null-CCM or *TPBG*-CCM. Representative microscopy images of the networks (**G**) and a bar graph showing the fold change of branch length vs Ad-Null (**H**). Scale bars=0.5 mm. All values are mean±SEM. ICC indicates immunocytochemistry. Unpaired *t* tests, **P*<0.05, ***P*<0.01, and ****P*<0.001 vs Ad-Null.

Increased Migratory and Proangiogenic Activities of TPBG-Transfected APCs Require CXCL12 Signaling Through CXCR7

We next asked whether CXCL12 is accountable for the functional effects induced by forced TPBG expression in human APCs. To this aim, *TPBG*-transduced APCs were cultured in EGM2 (endothelial growth medium 2) for 48 hours and then the CCM was collected, treated with CXCL12 inhibitor or isotype control for 1 hour, and finally used to condition APCs in scratch or Matrigel assays. CXCL12 neutralization resulted in an impairment of the migratory (Figure 5A and 5B) and network promotion capabilities of *TPBG*-transduced APCs (Figure 5C and 5D). To further determine the implicated receptor, *TPBG*-transduced APCs were exposed to a

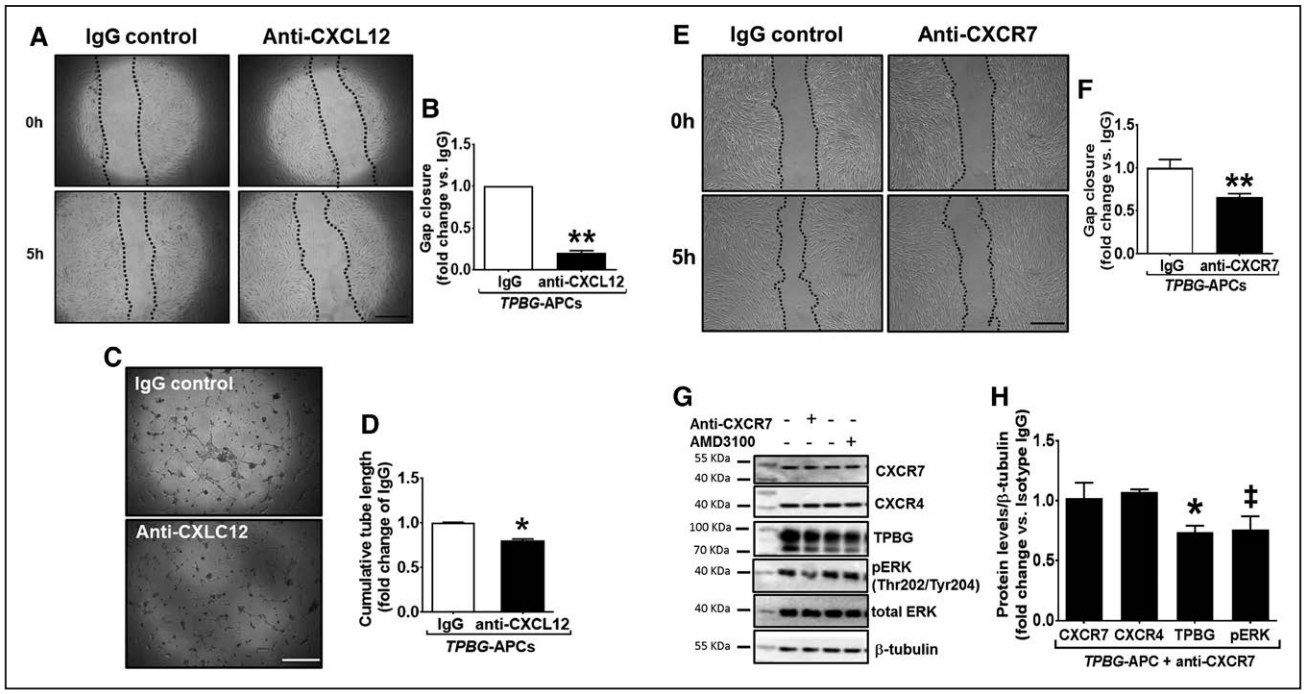

Figure 5. CXCL12 (chemokine [C-X-C motif] ligand-12) and CXCR7 (C-X-C chemokine receptor type 7) neutralization inhibits the migratory and network supporting activities of *TPBG*-transduced adventitial pericytes (APCs). **A** and **B**, Neutralization of CXCL12 reduces the migratory activity of *TPBG*-APCs. Representative images (**A**) and bar graphs illustrating the fold change vs IgG (**B**). **C** and **D**, *TPBG*-APCs cocultured with human umbilical cord endothelial cells (HUVECs) were assayed in a Matrigel assay in the presence of *TPBG*-cell conditioned medium (CCM) containing the CXCL12 neutralizing antibody. Representative images of the networks (**C**) and a bar graph illustrating the change in tube length (**D**). **E**–**H**, CXCR7 blockade impairs APC migration in association with lower levels of TPBG (trophoblast glycoprotein) and pERK. Representative images of the scratch assay (**E**) and bar graphs illustrating the fold change vs IgG (**F**). **G**, Representative Western blot images and (**H**) band densitometry data normalized by β-tubulin. Experiments were performed in quadruplicate or quintuplicate and repeated with 3 APC lines. Scale bars =0.5 mm. All values are mean±SEM. ANOVA was used to compare multiple groups and unpaired *t* tests to compare 2 groups, **P*<0.05 and ***P*<0.01 vs IgG. ‡, statistical trend as *P*<0.150 (*P*=0.064).

neutralizing anti-CXCR7 antibody or the CXCR4 antagonist AMD3100, followed by addition of their own 48 hours CCM. Isotype IgG or solvent controls were respectively used as controls. AMD3100 had no effect on migration (0.96±0.10-fold change, *P*=NS versus control). In contrast, CXCR7 neutralization impaired migration (0.66±0.04, *P*<0.05 versus control; Figure 5E and 5F). The 2 inhibitors did not alter the expression of CXCR4 and CXCR7 as assessed using Western blots (Figure 5G and 5H). Interestingly, blockade of CXCR7 was associated with lower levels of TPBG expression (0.74±0.05 TPBG/β-tubulin fold change, *P*<0.01versus Isotype IgG) and pERK/ERK (0.76±0.11 pERK/ERK fold change, *P*=0.06 versus Isotype control; Figure 5G and 5H). The latter effect is compatible with the inhibition of postreceptor signaling, while the former one may suggest the existence of a reciprocal cross-talk between TPBG and CXCR7.

Mechanisms Underpinning TPBG Transcription

A search of the literature on prospective transcriptional regulators identified CITED2 as a potential candidate for TPBG regulation. Transcript and protein levels of CITED2 were assessed at different time points following APC exposure to normoxia or hypoxia. *CITED2* mRNA levels showed a reduction after 12 and 24 hours of hypoxia (Figure VIIA in the online-only Data Supplement). In contrast, CITED2 protein levels were found significantly increased at 48 hours (Figure 6A and 6B). We next determined *TPBG* mRNA levels in APCs transfected with full-length pcDNA3-*CITED2* or the empty-pcDNA3 vector as a control (Figure 6C). *CITED2* forced expression increases the levels of *TPBG* transcript by 1.6-fold (*P*<0.001 versus empty vector; Figure 6D). We next performed ChIP assays to determine if CITED2 influences *TPBG* directly. CITED2 has been demonstrated to bind DNA via AP-2 (adaptor protein complex AP-2 subunit alpha-2).[31] Promoter prediction software indicated there were 5 potential AP-2 binding sites upstream of the *TPBG* transcriptional start site (<3000 kb) and one potential site within exon 2. ChIP assays confirmed the occupation of CITED2 at locations −1985/−1836 bp and +482/+555 bp as compared with IgG or no antibody controls (*P*<0.05; Figure 6E). Quantitative ChIP analysis showed a further enrichment of CITED2 at the above locations in APCs exposed to hypoxia (*P*<0.05 versus IgG; Figure 6F). Next, we investigated if CITED2 knocking down blunts *TPBG* expression and functional effects in hypoxia. A pool of 4 siRNA control (siCtrl) or siRNA *CITED2* was transfected at 70 nmol/L. Knocking down was confirmed at the mRNA level by qPCR throughout the experimental follow-up (*P*<0.001 versus siCtrl; Figure 6G). ICC was used to confirm the location of CITED2 within the nucleus (Figure VIIB in the online-only Data Supplement). A reduction of immunopositive CITED2 was evidenced. Moreover, TPBG was significantly downregulated (as seen by Western blot; Figure 6H and 6I). TPBG downregulation was reported after 48 to 72 hours by an overall 0.78±0.10-fold change, *P*<0.05 compared with the siCtrl. Scratch assays additionally showed that silencing of *CITED2* was associated with a lower migration profile in the

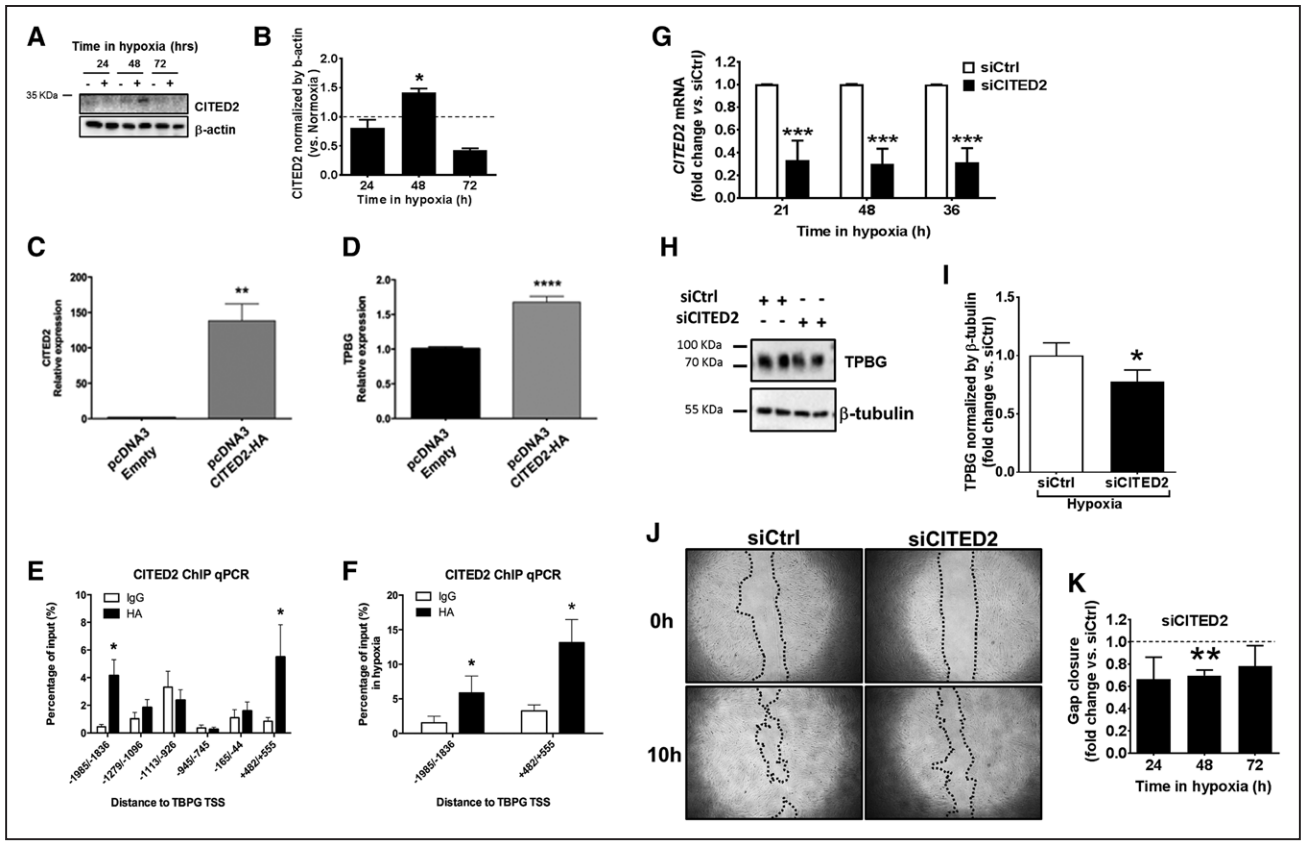

Figure 6. Transcriptional modulation of *TPBG* by CITED2 (Atypical chemokine receptor 3 CBP/p300-interacting transactivator with glutamic acid (E)/aspartic acid (D)-rich tail) in human adventitial pericytes (APCs). **A** and **B**, Hypoxic-dependent expression of CITED2 in 4 APC lines. **A**, Representative time course of CITED2 protein, with β-tubulin used as loading control. (–)=no hypoxic conditioning; (+)=hypoxic conditioning. **B**, Results of densitometry analysis. **C** and **D**, Forced *CITED2* expression (**C**) is associated with *TPBG* upregulation (**D**). APCs were transfected with either pcDNA3-Empty or pcDNA3-CITED2 tagged with human influenza hemagglutinin (HA) nonapeptide. *UBC* was used as a reference transcript (n=5 replicates). **E**, Quantitative ChIP-polymerase chain reaction (PCR) analysis of occupancy by CITED2 at 5 potential binding sites around *TPBG* transcriptional start site (TSS) APCs under normoxic conditions (n=5 replicates). **F**, Quantitative ChIP-PCR analyses of occupancy by CITED2 at –1985/–1836 bp and +482/+555 bp under hypoxic conditions, in the same cell lines. **G**–**K**, Knockdown of *CITED2* was performed to validate a regulatory effect on TPBG (trophoblast glycoprotein). Silencing was evidenced at the transcript level by quantitative PCR (qPCR; **G**). Each time point was statistically studied independently by unpaired *t* test. Four biological replicates were studied in technical duplicates or triplicates. All comparisons are done vs siRNA control (siCtrl). **H** and **I**, TPBG expression was diminished by siCITED2 as seen by Western blot. Representative immunoblot is displayed (**H**) and bar graphs have been used to represent band densitometry data (**I**). **J** and **K**, CITED2 silencing significantly diminishes APC migration after 48 h. Representative images are displayed in (**J**). Bar graph of gap closure with values calculated as fold change to overcome donor heterogeneity (**K**). SiCtrl conditions per each donor and time point were used as controls for each comparison. Dotted line represents the Gap closure fold change =1 of siCtrl. All values are mean±SEM. ANOVA was used to compare multiple groups and unpaired *t* tests to compare 2 groups, **P*<0.05, ***P*<0.01, and ****P*<0.001.

APCs, reaching the statistical significance after 48/72 hours (0.70±0.05, *P*<0.01; Figure 6J and 6K), a time that coincides with TPBG downregulation.

In Vivo Matrigel Plug Angiogenesis Assay With *TPBG*-Silenced APCs

Next, we performed gene silencing studies in mice, where the end point was the ability of TPBG to influence angiogenesis in vivo. In the first study, Matrigel plugs mixed with TPBG siRNA or control oligonucleotides were implanted subcutaneously. After 21 days, explanted TPBG-silenced plugs showed reduced microvascular networks compared with controls (*P*<0.05; Figure 7A and 7B), thus suggesting TPBG is essential for proper vascular formation.

Upregulation of *TPBG* in Resident Pericytes From Ischemic Muscles

To translate the data from in vitro hypoxia into a model of ischemia, we determined the expression levels of *TPBG* in vascular cells sorted from muscles of mice that underwent unilateral femoral artery occlusion. As shown in Figure VIII in the online-only Data Supplement, *Tpbg* mRNA levels were increased in CSPG4-sorted pericytes from ischemic muscles ($p < 0.05$ versus contralateral normoperfused muscles), whereas the expression of *Tpbg* in CD31-sorted ECs was not altered by ischemia. These data indicate that, as with in vitro hypoxia, in vivo ischemia induces the expression of Tpbg in murine pericytes.

Transplantation of *TPBG*-Transduced APCs in Mice With Limb Ischemia

We next determined if the forced expression of *TPBG* enhances the previously reported benefit of APC transplantation.[21,23,32] To this aim, we injected 100 000 human APCs, transduced with Ad-Null or Ad-*TPBG*, or vehicle intramuscularly in immunodeficient mice with limb ischemia (Figure 8A). The study protocol was a randomized treatment, with 2 independent operators assessing the outcomes

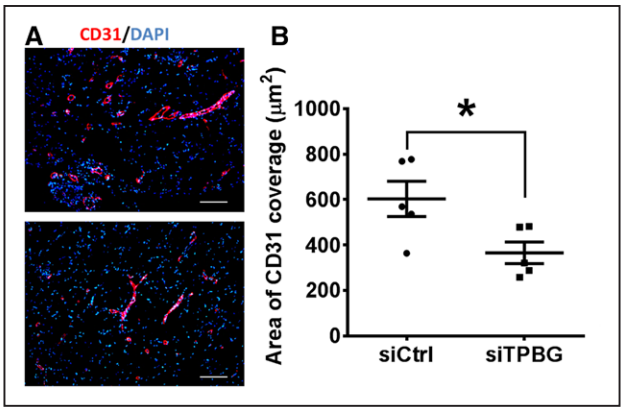

Figure 7. In vivo plug assay. **A**, Representative images showing the microvessels positive for CD31 (red) in the Matrigel plug. Five animals per group were studied. Scale bars, 50 μm. **B**, Quantification of the area of CD31 coverage in the Matrigel plugs mixed with *TPBG* siRNA (siTPBG) or control oligonucleotides at 21 d after implantation. Individual values and mean±SEM. Unpaired *t* test to compare 2 groups. DAPI indicates 4′,6-diamidino-2-phenylindole. **P*<0.05 vs siRNA control (siCtrl).

blind to the randomization codes. TPBG expression in APCs was verified before implantation by Western blotting of the c*Myc*-tag and assessment of total TPBG protein (Figure 8B and 8C).

More than 80% of the experimental subjects completed the study: 7/10 in the vehicle group, 8/10 in Ad-Null APC injected group, and 10/10 in the Ad-*TPBG* APC injected group. The different outcome was because mice from the vehicle- or the Ad-Null APC injected groups had to be humanely euthanized following the occurrence of toe necrosis, while none manifested these signs in the Ad-*TPBG* APC injected group. The χ^2 analysis denoted a difference in the above outcome between Ad-*TPBG* and control groups ($P<0.05$). APC transplantation accelerated blood flow recovery ($P<0.05$). The improvement in reperfusion was enhanced by Ad-*TPBG* APCs ($P<0.001$; Figure 8D and 8F). Ad-*TPBG* APCs induced an increase in capillary density as compared with Ad-Null APCs ($P<0.05$; Figure 8G and 8H). We could not evidence the presence of APCs in the ischemic muscles at 21 days post-transplantation, as assessed by targeting c-Myc (data not shown).

Discussion

Pericytes offer a tantalizing cell source for cardiovascular regenerative therapy.[21–23,32,33] In this study, we provide the first evidence that TPBG is expressed in pericytes in situ and after in vitro expansion, is upregulated in response to hypoxia, and is essential for migration and proangiogenic activities. We additionally identified the first *TPBG* transcriptional regulator to be described and demonstrated direct binding of CITED2 on the *TPBG* promoter region, which is further enhanced by hypoxia and participates in the migratory activity of APCs (Graphic Abstract).

In the placenta, TPBG is coexpressed with CITED2 by trophoblast cells and plays key roles in cell adhesion, migration, and proper capillary patterning.[34,35] Moreover, it has been demonstrated that hypoxia induces *CITED2* gene expression through HIF (hypoxia-inducible factor)1α-mediated transcriptional induction, followed by rapid and transient upregulation of CITED2 protein.[36–38] In turn, *CITED2* overexpression induces elevated *TPBG* transcripts,[39] and upregulates various components of the EMT-associated program, of which TPBG is an acknowledged member.[40,41] Haejin et al have proposed that CITED2 might be a regulator of HIF1α-dependent gene expression under hypoxic conditions.[38] CITED2 attenuates the activation of hypoxia-inducible genes by HIF1α through interference with the recruitment and interaction of HIF1α with p300. In the present study, we report that CITED2 associates with *TPBG* genomic regions at 2 AP-2 binding sites, consequently increasing TPBG transcription, and that binding affinity is enhanced by hypoxia. *CITED2* knocking down further supports those findings and reveals the CITED2's modulatory effect on APC migration, as assessed using a scratch assay.

This investigation in adult human vascular cells expands the information on CITED2 and TPBG available from developmental models. *Cited2* knockout is lethal, leading to a range of defects including malformed outflow tract, aortic-arch malformation, and abnormal microvessel development, which is thought to be a consequence of Cited2 regulation of Vegfa.[42–44] Furthermore, Cited2 activity influences proliferation and patterning of placental capillaries, with Cited2 null placentas displaying a disorganized pericyte arrangement in the capillaries.[35] Within human cardiovascular pathology, mutations of CITED2 and epigenetic modifications of its promoter region have been linked with congenital heart disease.[43,45,46] Tpbg knockout mice are viable, but adult animals show structural disorganization within the brain and exhibit a high frequency of hydrocephalus, requiring termination at early stages of the life.[1] Interestingly, recent studies suggest that the dysfunction of cellular constituents of the blood-brain barrier, including pericytes, may contribute in hydrocephalus.[47] Our discovery of an association between CITED2 and TPBG in human pericytes calls for new investigation on TPBG playing a pathogenic role in the above cardiovascular and brain defects.

We found several EMT-associated proteins, including TPBG, are induced in APCs under hypoxia (Figure 1; Figure V in the online-only Data Supplement). According to the emerging concept of EMT, stimulated epithelial cells may undergo a transformative process resulting in the acquisition of mesenchymal phenotypes endowed with migratory and proangiogenic properties. Here, we propose that, in analogy with EMT, hypoxia activates TPBG and associated partners to transform quiescent pericytes into migratory and proangiogenic pericytes. Furthermore, we show that CXCL12 signaling is key in this transformative phenomenon. Studies in embryonic cells indicate the transient presentation of TPBG at the plasma membrane during differentiation allows CXCL12 signal transduction through CXCR4.[3] Translating the above scheme into our experimental setting, we found hypoxia-induced TPBG expression was associated with a significant increase in CXCL12 transcription and secretion and reduction in DPP4 release. Hypoxia did not affect the expression of the canonical CXCR4 receptor but changed its subcellular location to the nucleus. This was associated with increased levels of CXCR7, the formation of CXCR7/CXCR4 heterodimer complexes and phosphorylation of ERK. These are typical

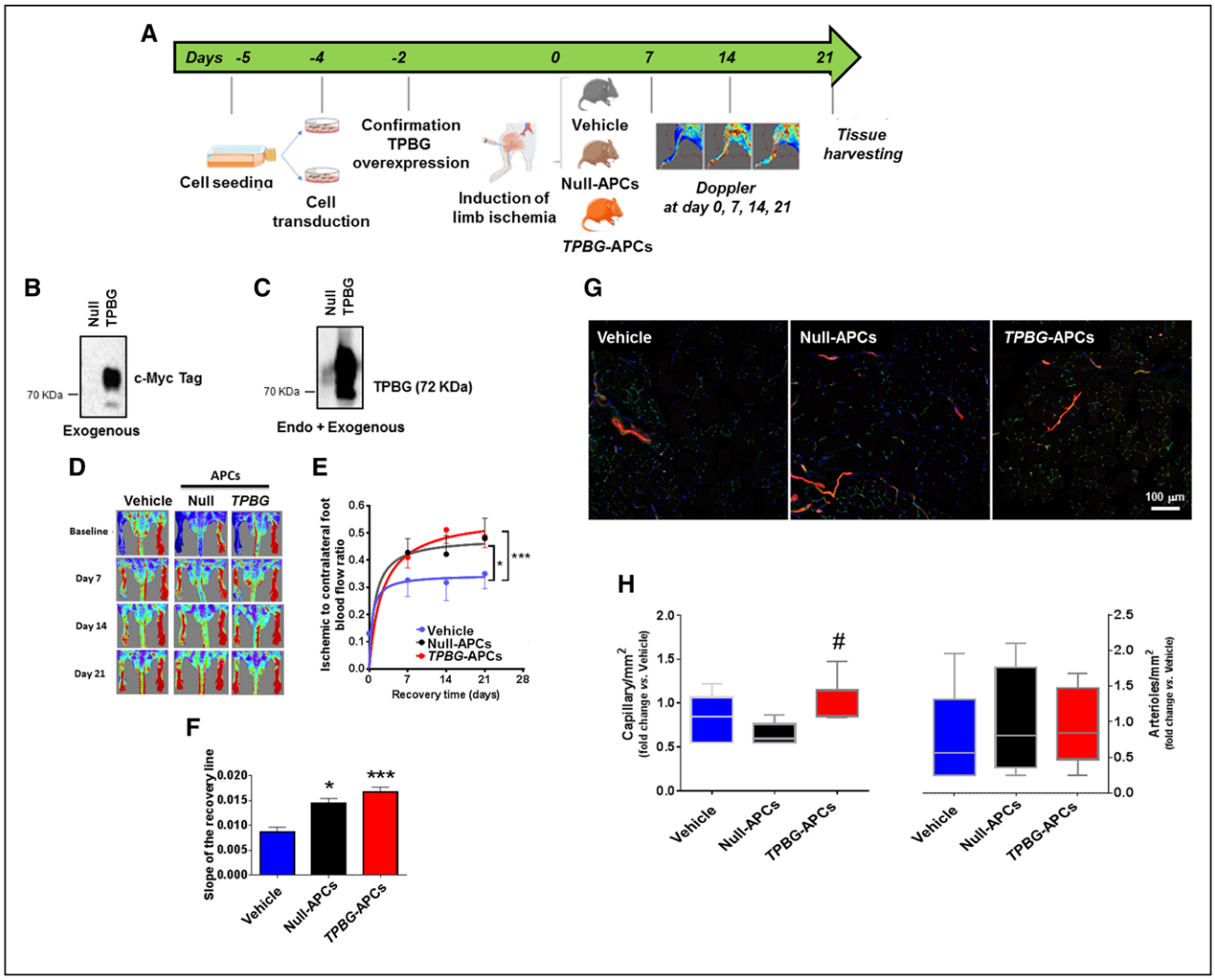

Figure 8. *TPBG*-transduced adventitial pericytes (APCs) improve recovery from limb ischemia. **A**, Mice were randomized to 3 treatment groups (n=7–10 each). APCs were transduced with an adenoviral construct containing c-Myc-tagged *TPBG* (Ad-*TPBG*) or an Ad-Null (moi 12) and injected in the ischemic adductor muscles after femoral artery ligation. A third group was injected with vehicle. Doppler imaging was performed at days 0, 7, 14, and 21. **B** and **C**, The overexpression of TPBG (trophoblast glycoprotein) was demonstrated by targeting the tagged sequence c-Myc (**B**). Representative blot showing the total amount of TPBG, including exogenous and endogenous (**C**). **D–F**, Representative images of laser Doppler blood flowmetry at baseline and during recovery (**D**). Graph showing the recovery pattern in the 3 study groups illustrated as nonlinear regression fits and mean±SEM for each time point (**E**). Bar graph showing slope values of recovery regression lines (**F**; **P*<0.05 and ****P*<0.001 vs vehicle; missing not at random [not ignorable] data from animals prematurely sacrificed because of the appearance of necrotic toes were imputed according to the worst scenario (the lowest value of the series). **G** and **H**, Immunohistochemistry assessment of vascular density performed in abductor muscles harvested from the ischemic left limb. IB4 (isolectin B4; green) and α-SMA (alpha smooth muscle actin; red) were immunostained to identify capillaries and arterioles. All samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Representative images (**G**) and box and whiskers graphs showing minimum and maximum values (**H**; #*P*<0.05 vs Ad-Null APCs). ANOVA was used to compare multiple groups and unpaired *t* tests to compare 2 groups.

modifications seen during acquisition of a migratory phenotype in cancerous cells.[1]

To establish the cause-effect relationship between TPBG and the CXCL12 migratory signaling, we performed gene titration studies using *TPBG* siRNA and an overexpressing vector in functional assays (Figures 3 and 4). Results of silencing confirm that TPBG is essential for the operativity of the CXCL12 migratory and angiogenic program in human APCs. As seen under hypoxia, forced *TPBG* expression induced a promigratory phenotype, which was associated with the formation of CXCR7/CXCR4 heterodimers and ERK phosphorylation. All these effects could be contrasted by CXCL12 neutralization or CXCR7 blockade. Altogether, these data suggest that CXCR7, which has been previously considered a decoy receptor, plays important functional roles in key activities of pericytes. Moreover, the murine angiogenesis plug assay model further validated the role of TPBG in postnatal neovascularization.

Preconditioning and genetic modification of donor cells have been considered as a potential strategy to increase resistance to ischemic stress, ultimately boosting therapeutic efficacy.[48–50] Accordingly, our study shows that, although resident pericytes already provide an endogenous source of TPBG under ischemia, transplantation of TPBG-overexpressing APCs improved the hemodynamic recovery and reparative capillarization compared with spontaneous responses observed in control mice. A puzzling aspect of data in the Ad-Null APC injected group is represented by the improvement in blood

flow despite no change in vascular density. A possible explanation is that cell therapy produced an increase in the size of collaterals in proximity to the artery occlusion, which could not be detected by the assessment of microvascular density in muscles. Intriguingly, we observed TPBG-overexpressing APCs induce a clinical improvement in toe necrosis as compared with Ad-Null APCs or vehicle, which indicate a positive effect on cicatrization and re-epithelialization. We speculate that this benefit is the result of paracrine and autocrine effects of upregulated CXCL12 signaling. In line with our findings, a recent publication has demonstrated that in situ bioengineered CXCL12 delivery enhances wound healing in different pathological scenarios including murine models of peripheral ischemia and hyperglycemia and wound healing of human skin models.[51] The rationale of using a gain of function approach in the in vivo studies was 2-fold. First, our research on APCs has a translational goal with the ultimate intention to find solutions for the treatment of ischemic disease. Second, although recognizing the mechanistic value of silencing therapeutic genes to obtain proof of concept, we were reluctant to use an approach that might be harmful to the critical limb ischemia model for reasons intrinsic to the guidelines of our ethical license.

In conclusion, the present study pinpoints TPBG as a novel mechanistic modulator of pericyte functions instrumental to vascular physiology and repair. Targeting TPBG may have, therefore, a relevant impact on regenerative therapies.

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Disclosures

None.

References

1. Southgate TD, McGinn OJ, Castro FV, Rutkowski AJ, Al-Muftah M, Marinov G, Smethurst GJ, Shaw D, Ward CM, Miller CJ, Stern PL. CXCR4 mediated chemotaxis is regulated by 5T4 oncofetal glycoprotein in mouse embryonic cells. *PLoS One.* 2010;5:e9982. doi: 10.1371/journal.pone.0009982
2. Christopherson KW II, Hangoc G, Mantel CR, Broxmeyer HE. Modulation of hematopoietic stem cell homing and engraftment by CD26. *Science.* 2004;305:1000–1003. doi: 10.1126/science.1097071
3. McGinn OJ, Marinov G, Sawan S, Stern PL. CXCL12 receptor preference, signal transduction, biological response and the expression of 5T4 oncofoetal glycoprotein. *J Cell Sci.* 2012;125(pt 22):5467–5478. doi: 10.1242/jcs.109488
4. Burger JA, Kipps TJ. CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. *Blood.* 2006;107:1761–1767. doi: 10.1182/blood-2005-08-3182
5. Busillo JM, Benovic JL. Regulation of CXCR4 signaling. *Biochim Biophys Acta.* 2007;1768:952–963. doi: 10.1016/j.bbamem.2006.11.002
6. Pawig L, Klasen C, Weber C, Bernhagen J, Noels H. Diversity and interconnections in the CXCR4 chemokine receptor/ligand family: molecular perspectives. *Front Immunol.* 2015;6:429. doi: 10.3389/fimmu.2015.00429
7. Rajagopal S, Kim J, Ahn S, Craig S, Lam CM, Gerard NP, Gerard C, Lefkowitz RJ. Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc Natl Acad Sci USA*. 2010;107:628–632.
8. Boldajipour B, Mahabaleshwar H, Kardash E, Reichman-Fried M, Blaser H, Minina S, Wilson D, Xu Q, Raz E. Control of chemokine-guided cell migration by ligand sequestration. *Cell.* 2008;132:463–473. doi: 10.1016/j.cell.2007.12.034
9. Luker KE, Steele JM, Mihalko LA, Ray P, Luker GD. Constitutive and chemokine-dependent internalization and recycling of CXCR7 in breast cancer cells to degrade chemokine ligands. *Oncogene.* 2010;29:4599–4610. doi: 10.1038/onc.2010.212
10. Naumann U, Cameroni E, Pruenster M, Mahabaleshwar H, Raz E, Zerwes HG, Rot A, Thelen M. CXCR7 functions as a scavenger for CXCL12 and CXCL11. *PLoS One.* 2010;5:e9175. doi: 10.1371/journal.pone.0009175
11. Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, Penfold ME, Sunshine MJ, Littman DR, Kuo CJ, Wei K, McMaster BE, Wright K, Howard MC, Schall TJ. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med.* 2006;203:2201–2213. doi: 10.1084/jem.20052144
12. Sun Y, Cheng Z, Ma L, Pei G. Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *J Biol Chem.* 2002;277:49212–49219. doi: 10.1074/jbc.M207294200
13. Cheng ZJ, Zhao J, Sun Y, Hu W, Wu YL, Cen B, Wu GX, Pei G. beta-arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization, and this implicates multiple interaction sites between beta-arrestin and CXCR4. *J Biol Chem.* 2000;275:2479–2485.
14. Ward CM, Barrow K, Woods AM, Stern PL. The 5T4 oncofoetal antigen is an early differentiation marker of mouse ES cells and its absence is a useful means to assess pluripotency. *J Cell Sci.* 2003;116(pt 22):4533–4542. doi: 10.1242/jcs.00767
15. Spencer HL, Eastham AM, Merry CL, Southgate TD, Perez-Campo F, Soncin F, Ritson S, Kemler R, Stern PL, Ward CM. E-cadherin inhibits cell surface localization of the pro-migratory 5T4 oncofetal antigen in mouse embryonic stem cells. *Mol Biol Cell.* 2007;18:2838–2851. doi: 10.1091/mbc.e06-09-0875
16. Eastham AM, Spencer H, Soncin F, Ritson S, Merry CL, Stern PL, Ward CM. Epithelial-mesenchymal transition events during human embryonic stem cell differentiation. *Cancer Res.* 2007;67:11254–11262. doi: 10.1158/0008-5472.CAN-07-2253
17. Torreggiani E, Lisignoli G, Manferdini C, Lambertini E, Penolazzi L, Vecchiatini R, Gabusi E, Chieco P, Facchini A, Gambari R, Piva R. Role of slug transcription factor in human mesenchymal stem cells. *J Cell Mol Med.* 2012;16:740–751. doi: 10.1111/j.1582-4934.2011.01352.x
18. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest.* 2009;119:1420–1428. doi: 10.1172/JCI39104
19. Shenoy AK, Jin Y, Luo H, Tang M, Pampo C, Shao R, Siemann DW, Wu L, Heldermon CD, Law BK, Chang LJ, Lu J. Epithelial-to-mesenchymal transition confers pericyte properties on cancer cells. *J Clin Invest.* 2016;126:4174–4186. doi: 10.1172/JCI86623
20. Lu J, Shenoy AK. Epithelial-to-pericyte transition in cancer. *Cancers (Basel)*. 2017;9:E77.
21. Campagnolo P, Cesselli D, Al Haj Zen A, Beltrami AP, Kränkel N, Katare R, Angelini G, Emanueli C, Madeddu P. Human adult vena saphena contains perivascular progenitor cells endowed with clonogenic and

proangiogenic potential. *Circulation.* 2010;121:1735–1745. doi: 10.1161/CIRCULATIONAHA.109.899252
22. Gubernator M, Slater SC, Spencer HL, et al. Epigenetic profile of human adventitial progenitor cells correlates with therapeutic outcomes in a mouse model of limb ischemia. *Arterioscler Thromb Vasc Biol.* 2015;35:675–688. doi: 10.1161/ATVBAHA.114.304989
23. Katare R, Riu F, Mitchell K, Gubernator M, Campagnolo P, Cui Y, Fortunato O, Avolio E, Cesselli D, Beltrami AP, Angelini G, Emanueli C, Madeddu P. Transplantation of human pericyte progenitor cells improves the repair of infarcted heart through activation of an angiogenic program involving micro-RNA-132. *Circ Res.* 2011;109:894–906. doi: 10.1161/CIRCRESAHA.111.251546
24. Caporali A, Meloni M, Miller AM, et al. Soluble ST2 is regulated by p75 neurotrophin receptor and predicts mortality in diabetic patients with critical limb ischemia. *Arterioscler Thromb Vasc Biol.* 2012;32:e149–e160. doi: 10.1161/ATVBAHA.112.300497
25. Nadgir SV, Hensler HR, Knowlton ER, Rinaldo CR, Rappocciolo G, Jenkins FJ. Fifty percent tissue culture infective dose assay for determining the titer of infectious human herpesvirus 8. *J Clin Microbiol.* 2013;51:1931–1934. doi: 10.1128/JCM.00761-13
26. Bhattacharya S, Michels CL, Leung MK, Arany ZP, Kung AL, Livingston DM. Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. *Genes Dev.* 1999;13:64–75.
27. Elsby LM, O'Donnell AJ, Green LM, Sharrocks AD, Roberts SG. Assembly of transcription factor IIB at a promoter *in vivo* requires contact with RNA polymerase II. *EMBO Rep.* 2006;7:898–903. doi: 10.1038/sj.embor.7400767
28. Yu B, Wong MM, Potter CM, Simpson RM, Karamariti E, Zhang Z, Zeng L, Warren D, Hu Y, Wang W, Xu Q. Vascular stem/progenitor cell migration induced by smooth muscle cell-derived chemokine (C-C Motif) ligand 2 and chemokine (C-X-C motif) ligand 1 contributes to neointima formation. *Stem Cells.* 2016;34:2368–2380. doi: 10.1002/stem.2410
29. Mehrad B, Keane MP, Strieter RM. Chemokines as mediators of angiogenesis. *Thromb Haemost.* 2007;97:755–762.
30. Wang L, Wang Z, Yang B, Yang Q, Wang L, Sun Y. CXCR4 nuclear localization follows binding of its ligand SDF-1 and occurs in metastatic but not primary renal cell carcinoma. *Oncol Rep.* 2009;22:1333–1339.
31. Braganca J, Swingler T, Marques FIR, Jones T, Eloranta JJ, Hurst HC, Shioda T, Bhattacharya S. Human CREB-binding protein/p300-interacting transactivator with ED-rich tail (CITED) 4, a new member of the CITED family, functions as a co-activator for transcription factor AP-2. *J Biol Chem*. 2002;277:8559–8565.
32. Avolio E, Meloni M, Spencer HL, et al. Combined intramyocardial delivery of human pericytes and cardiac stem cells additively improves the healing of mouse infarcted hearts through stimulation of vascular and muscular repair. *Circ Res.* 2015;116:e81–e94. doi: 10.1161/CIRCRESAHA.115.306146
33. Chen CW, Corselli M, Péault B, Huard J. Human blood-vessel-derived stem cells for tissue repair and regeneration. *J Biomed Biotechnol.* 2012;2012:597439. doi: 10.1155/2012/597439
34. Stern PL, Brazzatti J, Sawan S, McGinn OJ. Understanding and exploiting 5T4 oncofoetal glycoprotein expression. *Semin Cancer Biol.* 2014;29:13–20. doi: 10.1016/j.semcancer.2014.07.004
35. Moreau JL, Artap ST, Shi H, Chapman G, Leone G, Sparrow DB, Dunwoodie SL. Cited2 is required in trophoblasts for correct placental capillary patterning. *Dev Biol.* 2014;392:62–79. doi: 10.1016/j.ydbio.2014.04.023
36. Freedman SJ, Sun ZY, Kung AL, France DS, Wagner G, Eck MJ. Structural basis for negative regulation of hypoxia-inducible factor-1alpha by CITED2. *Nat Struct Biol.* 2003;10:504–512. doi: 10.1038/nsb936
37. Yin Z, Haynie J, Yang XM, Han BG, Kiatchoosakun S, Restivo J, Yuan SY, Prabhakar NR, Herrup K, Conlon RA, Hoit BD, Watanabe M, Yang YC. The essential role of Cited2, a negative regulator for HIF-1 alpha, in heart development and neurulation. *Proc Natl Acad Sci USA*. 2002;99:10488–10493.
38. Yoon H, Lim JH, Cho CH, Huang LE, Park JW. CITED2 controls the hypoxic signaling by snatching p300 from the two distinct activation domains of HIF-1α. *Biochim Biophys Acta.* 2011;1813:2008–2016. doi: 10.1016/j.bbamcr.2011.08.018
39. Tien ES, Davis JW, Vanden Heuvel JP. Identification of the CREB-binding protein/p300-interacting protein CITED2 as a peroxisome proliferator-activated receptor alpha coregulator. *J Biol Chem.* 2004;279:24053–24063. doi: 10.1074/jbc.M401489200
40. Chou YT, Hsieh CH, Chiou SH, Hsu CF, Kao YR, Lee CC, Chung CH, Wang YH, Hsu HS, Pang ST, Shieh YS, Wu CW. CITED2 functions as a molecular switch of cytokine-induced proliferation and quiescence. *Cell Death Differ.* 2012;19:2015–2028. doi: 10.1038/cdd.2012.91
41. Zhang L, Huang G, Li XW, Zhang YJ, Jiang Y, Shen JJ, Liu J, Wang QL, Zhu J, Feng XB, Dong JH, Qian C. Hypoxia induces epithelial-mesenchymal transition via activation of SNAI1 by hypoxia-inducible factor-1 alpha in hepatocellular carcinoma. *BMC Cancer*. 2013;13:108.
42. MacDonald ST, Bamforth SD, Bragança J, Chen CM, Broadbent C, Schneider JE, Schwartz RJ, Bhattacharya S. A cell-autonomous role of Cited2 in controlling myocardial and coronary vascular development. *Eur Heart J.* 2013;34:2557–2565. doi: 10.1093/eurheartj/ehs056
43. Li Q, Pan H, Guan L, Su D, Ma X. CITED2 mutation links congenital heart defects to dysregulation of the cardiac gene VEGF and PITX2C expression. *Biochem Biophys Res Commun.* 2012;423:895–899. doi: 10.1016/j.bbrc.2012.06.099
44. Lopes Floro K, Artap ST, Preis JI, Fatkin D, Chapman G, Furtado MB, Harvey RP, Hamada H, Sparrow DB, Dunwoodie SL. Loss of Cited2 causes congenital heart disease by perturbing left-right patterning of the body axis. *Hum Mol Genet.* 2011;20:1097–1110. doi: 10.1093/hmg/ddq554
45. Liu Y, Wang F, Wu Y, Tan S, Wen Q, Wang J, Zhu X, Wang X, Li C, Ma X, Pan H. Variations of CITED2 are associated with congenital heart disease (CHD) in Chinese population. *PLoS One.* 2014;9:e98157. doi: 10.1371/journal.pone.0098157
46. Xu M, Wu X, Li Y, Yang X, Hu J, Zheng M, Tian J. CITED2 mutation and methylation in children with congenital heart disease. *J Biomed Sci.* 2014;21:7. doi: 10.1186/1423-0127-21-7
47. Guerra M, Blázquez JL, Rodríguez EM. Blood-brain barrier and foetal-onset hydrocephalus, with a view on potential novel treatments beyond managing CSF flow. *Fluids Barriers CNS.* 2017;14:19. doi: 10.1186/s12987-017-0067-0
48. Katare R, Stroemer P, Hicks C, Stevanato L, Patel S, Corteling R, Miljan E, Vishnubhatla I, Sinden J, Madeddu P. Clinical-grade human neural stem cells promote reparative neovascularization in mouse models of hindlimb ischemia. *Arterioscler Thromb Vasc Biol.* 2014;34:408–418. doi: 10.1161/ATVBAHA.113.302592
49. Lan F, Liu J, Narsinh KH, Hu S, Han L, Lee AS, Karow M, Nguyen PK, Nag D, Calos MP, Robbins RC, Wu JC. Safe genetic modification of cardiac stem cells using a site-specific integration technique. *Circulation.* 2012;126(11 suppl 1):S20–S28. doi: 10.1161/CIRCULATIONAHA.111.084913
50. Nowakowski A, Walczak P, Lukomska B, Janowski M. Genetic engineering of mesenchymal stem cells to induce their migration and survival. *Stem Cells Int.* 2016;2016:4956063. doi: 10.1155/2016/4956063
51. Vågesjö E, Öhnstedt E, Mortier A, Lofton H, Huss F, Proost P, Roos S, Phillipson M. Accelerated wound healing in mice by on-site production and delivery of CXCL12 by transformed lactic acid bacteria. *Proc Natl Acad Sci USA*. 2018;115:1895–1900. doi: 10.1073/pnas.1716580115

Highlights

- TPBG (trophoblast glycoprotein) is abundantly expressed in human pericytes following exposure to hypoxia or ischemia and this is associated with upregulation and secretion of CXCL12 (chemokine [C-X-C motif] ligand-12)/CXCR7 (C-X-C chemokine receptor type 7) and pERK, resulting in activation of migration and angiogenesis.
- CITED2 (Atypical chemokine receptor 3 CBP/p300-interacting transactivator with glutamic acid (E)/aspartic acid (D)-rich tail) acts as a transcriptional regulator of TPBG, especially under hypoxic conditioning.
- The CITED2/TPBG/CXCL12 axis provides the platform for a transition of pericytes from a quiescent to an activated state instrumental to tissue repair.